Investigating effect of oxidative stress during in vitro myogenic differentiation

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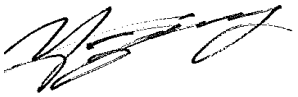

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Abstract

Stem cell research has been developing new ways to understand the mechanism behind aging in muscles as well as ways to prevent debilitating symptoms of it. In this study, *Pax7-CreER-Sod1 floxed* cells were used to model aging. 4-Hydroxy-Tamoxifen was used to activate the knockout mechanism in the floxed cells. The absence, or knockout, of the Sod1 gene contributes to the inability to break down reactive oxidative species. In vitro, the oxidative stress on the culture not only affects proliferation, but also affects differentiation of myoblasts. Increased 4-OHT concentration led to decreased nuclei quantity, myotube formation, and fusion index. While research linking “young” and “aged” circulatory systems has been done in vivo, this research aims to understand the fusion of “aged” and “young” cells in vitro. The breakdown of oxidative species is important for increased cell division, myotubule formation, and muscle fiber formation. More research must be conducted to identify the mechanism behind this behavior such as quantifying reduced oxidative stress with the contribution of Sod1-expressing cells in Sod1-null cultures.

Keywords: Muscle stem cell, Sod1 gene knock out, reactive oxidative species, myotube, myoblast

Introduction

Oxidative stress has been recognized in being one of the main factors of neuromuscular diseases, and other age-related damages [1-4]. Cu/Zn superoxide dismutase (also known as Sod1) is an antioxidant enzyme that remove products of reactive oxidative species (ROS) that induce oxidative stress [2, 4, 5]. Therefore, genetically modified SOD1 knockout (Sod1 KO) mice were studied to reveal the relation between aging and oxidative stress. In Hiroshi Nagahisa's study on skeletal muscle fibers of Sod1 KO mice, it was not only shown that Sod1 KO mice had lower body weights than the control, but there were increases in myonuclear frequency in all fiber types, mainly Type IIx/b which showed a decrease in muscle fiber population. When analyzing the mRNA expression, there were differences in transcription factor expression in those responsible for proliferation and differentiation (activated due to the lack of stem cells): MHC-e, Pax7, and MyoD had enhanced expression in the Sod1 KO mice; however, satellite cell frequency decreased similar to that of aging overall as seen on the Type Ia muscle fiber [1]. It is proposed that as ROS increases, satellite cell count decreases due to loss of functional proliferation. This leads to an increased FGF2 and IL-6 production, which enhances MyoD, myogenin and MHC-e expression in hopes of increasing satellite cell count [1]. In Yiqiang Zhang's study on the relationship between oxidative stress in Sod1 KO mice and cell senescence, it was shown that Sod1 KO mice do indeed have higher rates of cell senescence by observing dietary-restricted mice had increased lifespans and reduced levels of DNA oxidation [4]. This study provides a potential explanation as to why Sod1 KO mice, which have increased oxidative stress and therefore increased cell senescence, show signs of aging faster than the control mice. However, this is a specific conclusion as this would only apply to Sod1 KO mice who show increased cell senescence which aligns with Aphrodite Vasilaki's study that ROS alone may not be as directly damaging to muscles over time [5]. These studies again reinforce the idea that oxidative stress has an important role while aging and Sod1 plays as a key enzyme for removing oxidative stress.

Besides oxidative stress, stem cells attribute significantly in aging. For muscle tissue, Pax7 positive satellite cells (also known as muscle stem cells) maintain tissue homeostasis by proliferation and differentiation. Previous studies indicated that the mechanism of stem cell proliferation and differentiation being dependent on multiple factors such as niche, oxidative stress, and nutrient availability, but the overarching issue has been the control of these mechanisms in different environments. As mentioned, successful muscle regeneration is dependent on the environment the cells reside in [6]. To test this, genetic and niche-altering conditions have been explored. Gene targeting techniques utilizing markers for Pax3 and Pax7 have proved essential in studying this mechanism as Pax3 transcription factors are responsible for limb and diaphragm muscle generation while lack of Pax7 transcription factors are indicative of poor postnatal muscle generation [6]. In a study conducted by Christoph Lepper, Pax7-expressing satellite cells are critical in repairing damaged muscle tissue post-injury. The satellite cell-derived myoblasts differentiate into myofibers upon transplantation into a single muscle fiber or minced mice muscles; however, whether these Pax7+ cells are the only progenitors for muscle fiber regeneration was in question, and it was tested to see whether other cells (Pax7-) could induce repair as well [7]. It was concluded that Pax7+ cells are indeed crucial to muscle regeneration as experiments with induced Pax7+ cell elimination via tamoxifen proved fatal as the mice typically died within about a week of tmx-administration [7]. This did not conclude that Pax7- satellite cells were unimportant, but rather emphasized the importance of satellite cells for muscle repair. The mice used in this experiment were about 3-4 months old, a fairly young sample.

Although these satellite cells may be abundant in younger animals, studies show that there is a significant decline in satellite cell number per myofiber in aged animals and was found that this could be a result of decreases in β-gal protein expression combined with limited proliferation ability of aged satellite cells [8]. However, in age-modeling mice such as Sod-/-, Pax7 expression increased most likely due to a compensatory behavior following increased oxidative stress [1, 9]. Although there may have been an increase in expression of the transcription factor, many symptoms related to aging have been recorded. The mechanism behind this increase has not yet been explored, but this gives insight that age-related regeneration inhibition is not autonomous. Furthermore, the way muscle fibers are formed in the presence of competent and incompetent muscle stem cells has not been explored. As a result, when working with limited adult cells, the problems faced include functionality more than proliferation of *in vitro* cell culture methods of muscle stem cell expansion and enhanced regeneration in aged or damaged muscle stem cells.

Although the literature provides the mechanisms for Sod1 expression in healthy muscles and potential mechanisms in unhealthy muscles, there have not been experiments performed culturing Sod1 KO cells and normal cells together to see how muscle fibers will form in the presence of healthy and unhealthy satellite cells. This current research will review the potential effects of Sod1 KO cells on wildtype cells in terms of proliferation and differentiation. The myogenic differentiation abilities will be quantified by the fusion index. The number of Sod1 KO cells and WT cells will be compared in one single myotube. However, culturing myoblasts isolated from Sod1 KO mice has shown to be an issue in past experiments because of its functional debilitations. Therefore, in this study, we will utilize controlled Sod1 KO method that using Pax7-CreER-Sod1 flox system. After administration of tamoxifen, Cre recombinase will produced only in Pax7+ cells. As a result of removing loxp site, Sod1 protein will lose its function (knockout). For the convenience of experiment, myoblasts isolated from H2B-EGFP mice which has GFP tagging on the histone protein (nuclei tagging) used for WT cells.

To investigate effect of oxidative stress during in vitro myogenic differentiation and get an idea about whether "healthy" and "unhealthy" muscle stem cells can differentiate into a single muscle fiber or not, this study will culture Pax7-Sod1 flox treated with tamoxifen and Sod1(+/+) cells in the same dish and observe cell nuclei and cytoskeletons through fluorescent microscopy.

Material and methods

Isolation and purification of myoblast with preplating method

Pax7-Sod1 flox myoblasts were isolated and cultured from the Pax7 CreER-Sod1 flox mouse (3.26 months old), and wild-type myoblasts were isolated and cultured from H2B-EGFP mouse (1.69 months old). After euthanization of the mice, muscles were collected from the hind limb of each mouse in 2 small petri dishes with 3-5 mL of PBS. Muscles were minced to a paste using iris scissors. The paste was transferred into a tube with a digestion solution of 0.2% type II collagenase, Dulbecco's Modified Eagle Medium (DMEM) (5 mL per 11.1 mg of collagenase). 5 ml of digestion solution was used for each mouse tissue. The mixture was incubated at 37 ℃ for 60 minutes on a shaker. The minced tissue was then passed through a 20 G syringe needle several times. The homogenate was collected from the syringe and dissolved with 40 mL of PBS. This solution was then filtered with a 40-micrometer nylon strainer into a 50 mL conical tube. This was centrifuged at 500xg for 5 minutes. After discarding the supernatant, the tissue-cell mixture was suspended in 10 mL of growth media (DMEM

high glucose, 20% Fetal Bovine Serum, 1% pen/strep antibiotics).

The cell-containing growth media was transferred to a type I collagen-coated dish (prepared the night before for drying time) to pre-plate in the incubator overnight. Fibroblasts are shown to attach to collagen-coated dishes before myoblasts, so this step allows fibroblasts to attach to the dish first. About 16 hours later, the supernatant mixture with unattached myoblasts (and other substances) was pipetted onto a different type I collagen-coated dish (prepared the night before). This step also allowed additional fibroblasts to attach to the collagen-coated dish. The dish was placed in the incubator for 3 hours. The supernatant solution was then transferred to a dish coated with Matrigel (prepared 1hr before cell seeding) for 24 hours. Myoblasts are shown to attach to Matrigel better, so at this point, myoblasts were attaching to the dish. After the 24 hours, the debris and unattached cell solution was discarded and replaced with fresh growth media atop a dish with attached myoblasts. The cells on the Matrigel dish were detached with 3-5 mL of Trypsin/EDTA and incubated for 3-5 minutes, stopped with 5 mL of growth media and washed with PBS. In a conical tube, the detached cells were collected and centrifuged at 300xg for 5 minutes. After discarding the supernatant fluid, the cells were resuspended into fresh growth media. The cell containing growth media was then transferred to a new type I collagen dish. This is the final purification step. The dish was incubated for 5 minutes, to allow attachment of excess fibroblasts, then shaken or tapped to suspend potentially attached myoblasts. This shaking and attaching step was repeated at least 5 times. Finally, the unattached cells with growth media was transferred to another Matrigel-coated dish. After culturing isolated myoblasts on Matrigel coating for 2 days the cells were ready to be detached and used for the experiment. From the culture of H2B-EGFP cells, myotubes with multi nuclei can be observed through fluorescence microscope (Figure 1).

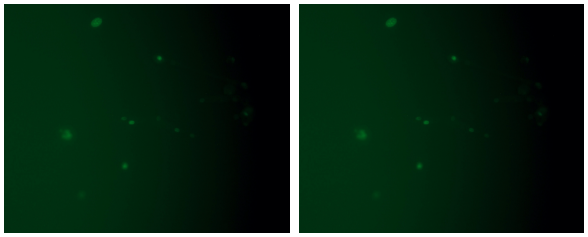


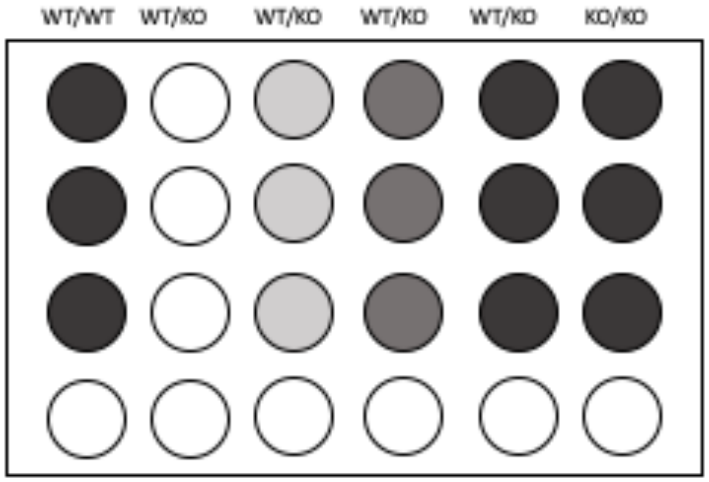
Figure 1. Representative image of differentiated myoblasts from H2B-EGFP mouse. Bright field image shows elongated and fused morphology of myotubes (Left panel). Green fluorescence from histone protein indicates nucleus. Multiple nuclei can be observed in. single myotube.

Experimental design for in vitro experiment

After detaching with Trypsin/EDTA, cells were counted using trypan blue staining and hemocytometer. After counting the total number of cells from the two dishes, cells were placed in the first 18 wells of a Matrigel-coated 24-well plate (Figure 2). The first six wells were considered Trial 1; the second group of six, Trial 2; and the final group of six, Trial 3. Each well in a single column should have the same number of cells with the same conditions. The first column had 16,000 H2B-GFP cells seeded onto each well with 250 µL of growth media. The second, third, fourth, and fifth column each had 8,000 H2B-GFP cells and 8,000 Pax7-Sod1 flox cells seeded onto each well with 250 µL of growth media. The final sixth column had 16,000 Pax7-Sod1 flox cells seeded onto each well with 250 µL of growth media. The first and sixth columns were used as control groups. The viability of the cells themselves may be checked with these two control columns.

Tamoxifen

After the cells attach to the dish (overnight), a tamoxifen/differentiation media solution (4-Hydroxy-Tamoxifen, DMEM high glucose, 5% Horse Serum, 1% pen/strep antibiotics) was treated to all wells in each column of the plate in this order of concentration:



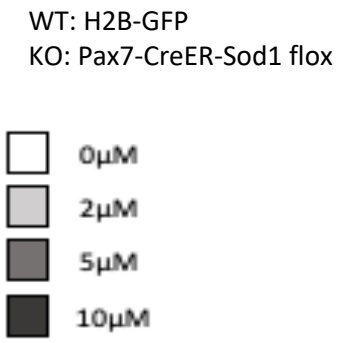


Figure 2. 24-well plate. 4-Hydroxy-Tamoxifen + differentiation media of varying concentrations. 8,000 cells each of respective mouse cell lines.
Total: 16,000 cells per well.

10 μM, 0 μM, 2 μM, 5 μM, 10 μM, 10 μM (*Fig. 2*). Studies have shown that tamoxifen effectively knocks out the gene at a concentration of 10 μM. The first and last column intended to show how tamoxifen affects the H2B-GFP cells in comparison to the Pax7-Sod1 flox cells. The order of ascending concentration of tamoxifen represents the degree of gene knockout in each well. For example, a 0 μM tamoxifen solution should not knock out the Sod1 gene; therefore, the Pax7-Sod1 flox cells do not have a change in oxidative stress. For the 5 μM tamoxifen solution, Pax7-Sod1 flox cells will experience some knock out which will theoretically result in a change in oxidative stress and fusion ability. We can assume that the 10 μM tamoxifen solution effectively knocked out the Sod1 gene in Pax7 satellite cells.

Culture and Differentiation

The cells were left in the 5% oxygen incubator to differentiate for a total of 3 days. Sod1-KO cells have been seen to die faster in 20% oxygen conditions; therefore, not only does 5% oxygen emulate the physiology of the human body, but it prevents the Sod1-KO cells from dying too quickly. As a note, the 5% oxygen incubator should not affect the Wild-type cells.

Imaging

The plate was imaged using a fluorescent microscope for 4 days: Day 0, Day 1, Day 2, Day 3. Day 0 represents the attached cells before tamoxifen treatment. Day 1 represents the first day of tamoxifen treatment. Wells from Day 0-2 were imaged using the regular TL-D microscope light. Cell death was observed throughout the days. On Day 3, cells were fixed with 4% PFA to be stained for fluorescent imaging. DAPI and Phalloidin-555 were used to stain the nuclei and cytoskeleton respectively. Using the program Zen, fluorescent and non-fluorescent images of the wells were taken to see how many myotubes formed by the nuclei of Pax7-Sod1 flox, H2B-GFP, and Pax7-S1 KO cells.

Results

Using the program Zen, snapshots were taken of wells at different time stamps. There shows increased cell death in column 1 (Wild-type/Wild-type, 10μM), column 3 (Wild-type/Knockout, 2μM), column 4 (Wild-type/Knockout, 5μM), column 5 (Wild-type/Knockout, 10μM), and column 6 (Knockout/Knockout, 10μM) throughout the days (*Fig. 3[a, c, d, e, f]*).

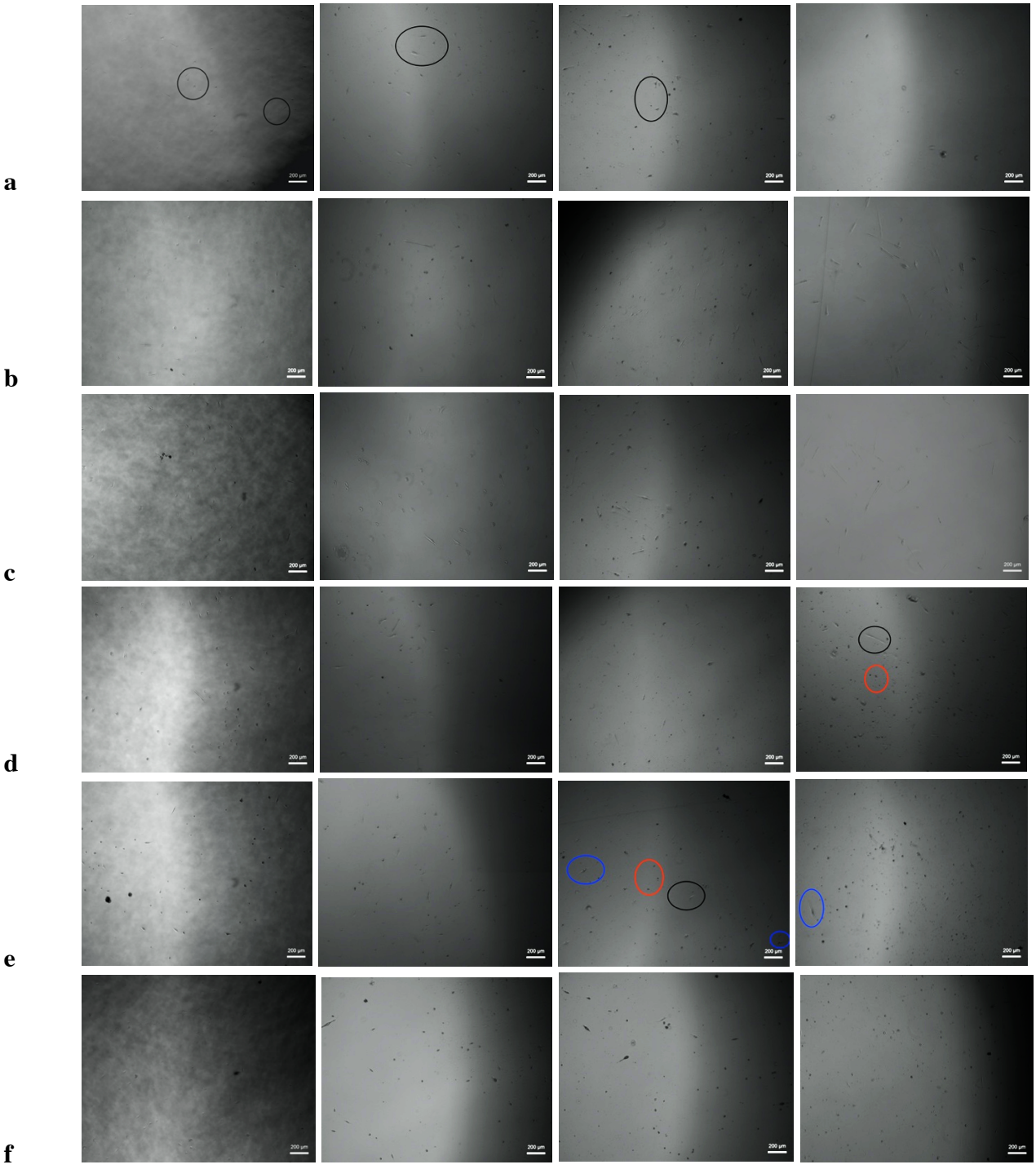


Figure 3. TL-D Light Images. **[a]** 16,000 H2B-GFP cells. 10μM wells chronologically ordered: Day 0, Day 1, Day 2, Day 3. **[b]** 8,000 H2B-GFP+8,000 Pax7-Sod1 flox/KO. 0μM: Day 0, Day 1, Day 2, Day 3. **[c]** 8,000 H2B-GFP+8,000 Pax7-Sod1 flox/KO. 2μM: Day 0, Day 1, Day 2, Day 3. **[d]** 8,000 H2B-GFP+8,000 Pax7-Sod1 flox/KO. 5μM: Day 0, Day 1, Day 2, Day 3. **[e]** 8,000 H2B-GFP+8,000 Pax7-Sod1 flox/KO. 10μM: Day 0, Day 1, Day 2, Day 3. **[f]** 16,000 Pax7-Sod1 flox/KO. 10μM: Day 0, Day 1, Day 2, Day 3.
Black circles indicate examples of attached myoblasts. Red circles indicate examples of dead myoblasts. Blue circles indicate myotubes.

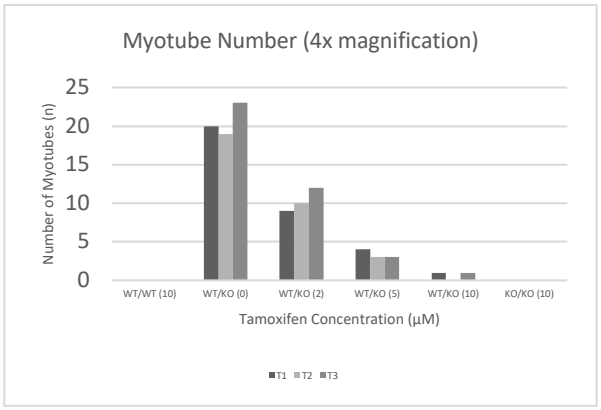


Figure 4. Myotube Number. Myotubes counted on 4x magnification for each trial (averaged). WT: H2B-GFP (1.69 mo); KO: Pax7-CreER-Sod1 flox (3.26 mo).

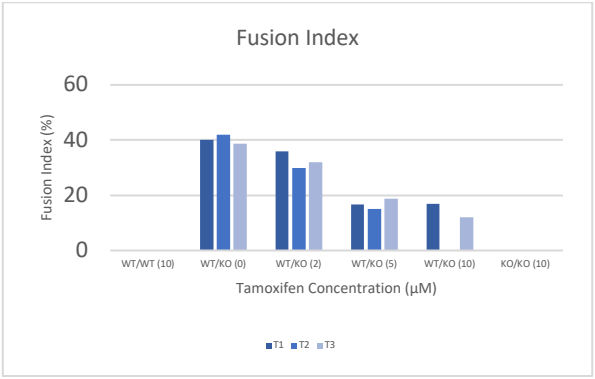


Figure 5. Fusion Index. Divided number of nuclei in myotubes by the total number of nuclei in the well. Counted on 4x magnification. WT: H2B-GFP (1.69 mo); KO: Pax7-CreER-Sod1 flox (3.26 mo).

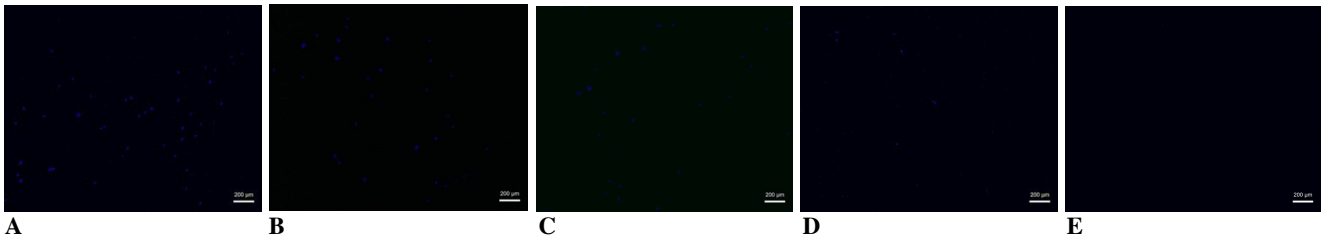


Figure 6. Nuclei. DAPI staining of Pax7-Sod1 flox nuclei. Both DAPI and EGFP signal exposures were increased. **[A]** 8,000 H2B-GFP+8,000 Pax7-Sod1 flox/KO. 0μM: Day 3. [**B]** 8,000 H2B-GFP+8,000 Pax7-Sod1 flox/KO. 2μM: Day 3. **[C]** 8,000 H2B-GFP+8,000 Pax7-Sod1 flox/KO. 5μM: Day 3. **[D]** 8,000 H2B-GFP+8,000 Pax7-Sod1 flox/KO. 10μM: Day 3. **[E]** 16,000 Pax7-Sod1 flox/KO. 10μM: Day 3.

All quantifications are based on the images taken, not the entire well. However, all images taken are the best representations of each well in a smaller scale.

The wells with higher tamoxifen concentration showed to have higher cell death. Following increased cell death, there was decreased myotube formation in all relevant columns. Column 1 shows the smallest number of myotubes while column 2 shows the largest number of myotubes in the given sample, and Column 5 shows more myotubes than 6. (*Fig. 4*).

For the Day 3 images*, average fusion indices were calculated per well for each column. The fusion index determines the number of nuclei in a single myotube in relation to the total number of nuclei in the well. Total nuclei quantity is shown to decrease with increased tamoxifen concentration (*Fig. 6[a-e]*). Column 6 shows the lowest fusion index while column 2 shows the highest fusion index (*Fig. 5*). Fusion indices are shown to decrease with increase in tamoxifen concentration (*Fig. 5*).

*Due to changes in the fluorescent microscope on 4/23/21, H2B-GFP/H2B-GFP could not be counted in figures 5, and 6.

Discussion

The Sod1 gene is necessary for breaking down reactive oxidative species and reducing oxidative stress. The study's results show the correlation between decreased Sod1 expression and decreased proliferation (*Fig. 3, 6*), myotube number (*Fig. 4*), and fusion index (*Fig. 5*). Each column with increased 4OHT concentration represents a related increase in percent knockout

of the Sod1 gene. The knockout of the Sod1 gene causes Cu/ZnSod malfunction which prevents reactive oxidative species from being broken down [12]. Therefore, it is assumed that the increase in 4OHT concentration causes an increase in oxidative stress. On Day 0, before 4OHT treatment, the cell population is relatively similar in number. At this point, it was assumed that there were 8,000 wildtype cells and 8,000 floxed cells in columns 2-5. However, due to the low viability of the wildtype cells, the mechanism of fusion between two different cell lines is inconclusive.

Pax7+ Satellite Cells

The Pax7-CreER-Sod1 flox mouse line has Sod1 floxed gene found only in the Pax7-expressing satellite cells. For in vitro purposes, this line works just as well as a regular Sod1 flox line as literature shows Pax7 expression to be critical in normal proliferation and differentiation [2].

Cell Viability

A crucial note of this study is the viability difference between the two cell lines. The H2B-GFP (Wild-type) cells had relatively low viability compared to the Pax7-Sod1 flox cells (*Fig. 3a, 3f*). The wildtype and floxed cells were seeded at the same time in the correct quantities, but more H2B-GFP cells died than the floxed cells. On Day 0, before tamoxifen treatment, H2B-GFP cells failed to attach to the dish and proliferate as quickly as the Pax7-Sod1 flox cells. Based on Control Column 1 (Wildtype/Wildtype, 10 μM), it is safe to assume that the H2B-GFP wild-type line did not contribute to the reduction in oxidative stress as much as expected. Instead, the percent knockout of Pax7-Sod1 flox cells contributed to the differences in cell death and fusion index. However, comparing column 5 (Wildtype/Knockout, 10 μM) and column 6 (Knockout/Knockout, 10 μM), there is a difference in fusion indices. This experiment would have to be repeated with cell lines of similar viabilities.

EGFP Signal

EGFP signals appeared to be weak during fluorescent imaging. This could have been due to the change in lamp settings during the duration of the experiment. Another factor could be that the signal was weak in the specific H2B-GFP mouse. To prevent this in the future, anti-GFP antibodies must be used to increase signals during immunocytochemistry.

Growth Phase

The growth phase was relatively short: less than 24hr. Due to time constraints, the experiment was accommodated to fit a one-week schedule. In the future, cells should be left to proliferate for two or more days before treating with tamoxifen or differentiating. Another method is to seed more than 8,000 cells each (or 16,000 cells per well). This would allow more cells to attach to the dish on Day 0.

Conclusion/Future Directions

It was initially hypothesized that in a single myotube, there will be more wildtype (Sod1 +/+) nuclei than knockout (Sod1 -/-) nuclei. Due to the low viability of the wildtype cells, it is not proven that wildtype cells reduce oxidative stress on “aged” cells. However, it is concluded that the increased knockout of the Sod1 gene in a culture, an increase in oxidative stress, contributes to the malfunction of muscle regeneration. Treatment of 4OHT on Pax7-Sod1 flox cells decreased myotube formation and cell viability: the two key components of calculating the fusion index. As myotubes fail to form among detaching myoblasts, muscle fibers would not form, and regeneration would not occur [3].

The comparison of column 5 (Wildtype/Knockout, 10 μM) and column 6 (Knockout/Knockout, 10 μM) suggest that H2B-GFP cells may have contributed to reducing oxidative stress. However, further experiments must be conducted, clearly visualizing the fusion between the two cell lines. Key points to consider

in order to repeat the experiment include ensuring similar viability between cell lines, accurate time stamps and a longer growth phase, and strengthening fluorescent signals before imaging. After successful completion of the in vitro study, an in vivo study should be conducted testing implanted stem cells.

Sod1 KO mice are important to study when observing aging. Floxed mice in particular are easier to manage because they provide a more controlled environment to manipulate the experiment without waiting for mice to age and increasing the chances of sudden mouse death. Studying the fusion between Sod1 KO cells, "aged" cells, and normal wildtype cells could answer the question of how "young blood" could reverse aging. Going forward with advancing technology, this research could be used to engineer cells that reduce oxidative stress to alleviate the pain of aging, muscular atrophy, and deteriorating muscle health.

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